Petit Plasmid: A Small, Versatile Antibiotic-Free Multi-Application DNA Vector Driving Biotech Innovation

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Table of Contents

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Introduction

DNA plasmids find wide-ranging applications in diverse fields of research, including studies related to inflammatory and autoimmune diseases [1], infectious diseases [2], therapeutic cancer vaccines [3,4] , gene therapy [5,6], cell therapy [6–8], production of recombinant and therapeutic proteins [9,10], and viral vector production [11–13].

The utilization of antibiotic resistance genes as selectable markers is discouraged due to the potential uptake of the plasmid by endogenous bacterial flora. This could lead to the emergence and dissemination of antibiotic-resistant microbes. The presence of prokaryotic sequences and CpG dinucleotides should be minimized in clinical applications. While these elements are useful for the plasmid's maintenance within bacterial hosts, they can trigger adverse immune reactions when introduced into mammalian cells [14].

To satisfy regulatory agencies recommendations and develop safer and more effective products, a variety of antibiotic-free plasmids and their corresponding host strains were developed [15–22].

Herein, I disclose novel antibiotic resistance gene-free DNA plasmids and provide comprehensive details about the generation of exemplary strains capable of supporting their maintenance and replication.

Green et al. introduced regulators called "Toehold Switches," which involve an RNA hairpin structure that sequesters both the Ribosome Binding Site (RBS) and a Start codon [23]. In the presence of a trigger RNA, which interacts with a complementary region located at the 5' region of the hairpin, the RBS is exposed, enabling ribosomes to initiate translation of the downstream sequence. Remarkably, a single pair of switch-trigger RNA led to a substantial average fold-activation exceeding 400.

Chappell et al. presented the concept of “small transcription activating RNAs” (STARs), designed to bind to a complementary region located upstream of a terminator or its 5' portion. This interaction prevents the formation of a terminating hairpin, thereby allowing the transcription of a downstream gene [24]. Notably, one specific pair of STAR-RNA target (AD1 A5/S5) exhibited a striking level of regulation, achieving a 94-fold activation. Remarkable levels of gene activation, reaching up to 9000-fold, were attained through the utilization of a computationally-optimized combination of STAR : hairpin RNA and a mutant green fluorescent protein with reduced half-life [25].

Westbrook et al. disclosed a “dual transcription-translation activator” having a near-background basal activity in the un-induced state yet displaying an average activation level of 923-fold upon the presence of small transcription activating RNAs (STARs) [26]. The STAR-inducible switch is called a “dual control activator” and comprises a terminator sequence encompassing a concealed ribosome binding site (RBS), a poly-A tail, and a downstream Start codon. When STARs are absent, a terminating hairpin structure forms, leading to transcription termination. The RBS is concealed within the hairpin, effectively preventing undesired translation initiation of the transcript. When present, STARs bind a complementary region of the terminator, preventing the formation of a terminating hairpin and subsequently exposing the RBS. This orchestrated interplay facilitates both transcription and translation of the downstream coding sequence.

By fusing designed toehold sequences with either artificial or natural terminator sequences, Hong et al. produced novel "switchable transcription terminators" (SWTs) [27]. Specific trigger RNAs hybridize with the toehold region as well as a portion of the terminator, effectively hindering the formation of a terminating hairpin, and enabling the uninterrupted elongation of transcription. It is noteworthy that a

SWT derived from T500 terminator displayed minimal leakage in the un-induced state and showcased an outstanding fold-activation of up to 879 upon induction.

When a bacterial strain is knocked-out for an important gene G, it can become auxotrophic with respect to a substrate S [28]. For example, G can be the gene argA, hisB, ilvA, leuB, lysA, metA, pheA, proA, thrC, trpC, or tyrA, while S corresponds to the amino acid L-arginine, L-Histidine, L-Isoleucine, L-Leucine, L-Lysine, L-Methionine, L-Phenylalanine, L-Proline, L-Threonine, L-Tryptophan, or tyrosine, respectively [28]. To achieve regulated expression in the presence of small transcription activating RNAs (STARs), a dual transcription-translation activator sequence could be inserted upstream of the important gene G. The resultant DNA construct can then be integrated into the genome of a bacterial strain or its auxotrophic variants, leading to the generation of engineered conditionally-auxotrophic strains.

The antibiotic marker-free plasmids disclosed herein are called “Petit Plasmids” and comprise at least a STAR cassette and an origin of replication sequence. STAR cassette comprises a prokaryotic promoter, a STAR-encoding gene, and a prokaryotic terminator—all required for producing STAR transcripts. The origin of replication sequence (ori) is required for plasmid maintenance within a bacterial host. Exemplary origin of replication sequences include the minimal R6Kγ, which necessitates the in-trans expression of the π protein for plasmid replication [29–31], and colE2-P9, which relies on the parA for plasmid replication [32–34]. When this plasmid is introduced in an engineered conditionally-auxotrophic strain of the present disclosure, STARs are produced, the important gene G is unrepressed, and the substrate S becomes optional for growth. In the absence of the plasmid and without supplementing the culture medium with S, bacterial growth will either be inhibited or the cells will die.

Design

Petit Plasmid comprises a STAR cassette, serving as a selectable marker, and an origin of replication sequence, necessary for the plasmid’s maintenance and replication within a suitable bacterial host. A STAR cassette comprises a promoter, a STAR-encoding sequence, and a terminator. We will henceforth refer to the transcripts produced from the STAR cassette as “activating RNAs”. They bind a complementary region of an RNA sequence, hereafter referred to as an “RNA switch”. The interaction of an activating RNA with a complementary region of an RNA switch results in transcription elongation that subsequently leads to the translation of the nascent protein-encoding RNA sequence.

Pairs of “activating RNA” and “RNA switch” for use in the present disclosure can be sourced from Ref [23–27], patents WO2015161060A1 and WO2014074648A2.

Production of activating RNAs can be tuned by varying the strength of the promoter, which can be selected from various sources such as Chris Anderson’s collection of promoters, iGEM’s BIOFAB collection, or those disclosed in Ref [35,36]. Without being bound to a particular theory or mechanism, a very weak promoter will lead to the production of a small amount of activating RNA, thus favoring the growth of bacteria bearing multiple copies of Petit Plasmids. The promoter can further comprise an insulator in its 5’ terminal to minimize the effects of transcriptional interference caused by a cryptic promoter sequence located upstream of the STAR cassette (for detail, see Ref [37]).

A chromosomally integrated DNA cassette known as "Activating RNA-Inducible Gene of Interest" (ARIGoI), illustrated in Figure 1, encodes the RNA switch and the protein-encoding RNA sequence. Its antibiotic resistance and counter-selectable marker cassettes (SacB) can be removed from the sequence by using Flp

recombinase-mediated excision. The result is illustrated in Figure 2. The effects of the presence or absence of "activating RNAs" as well as supplementing or omitting the "substrate S" on bacterial cells growth are summarized in Figure 3. For streamlining integration processes, additional cassettes, such as those encoding π protein or parA protein (replication gene), can be inserted into ARIGoI. The construct is illustrated in Figure 4.

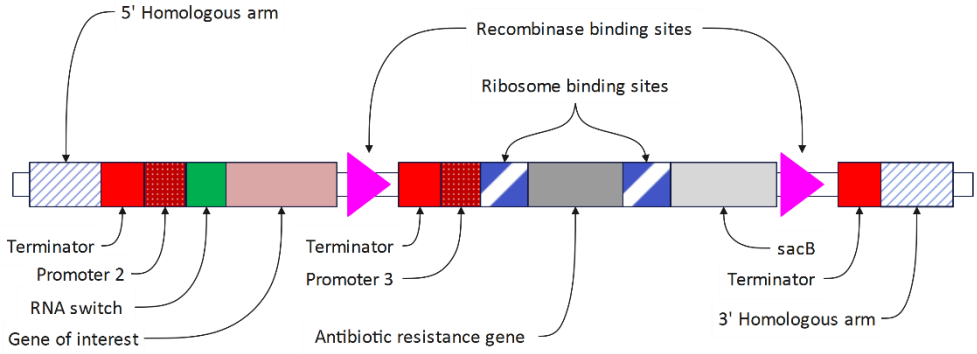


Figure 1: Components of "Activating RNA-Inducible Gene of Interest" (ARIGoI).

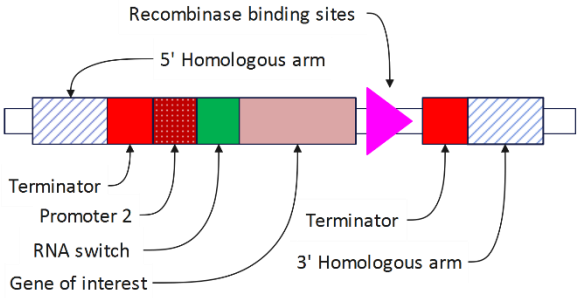


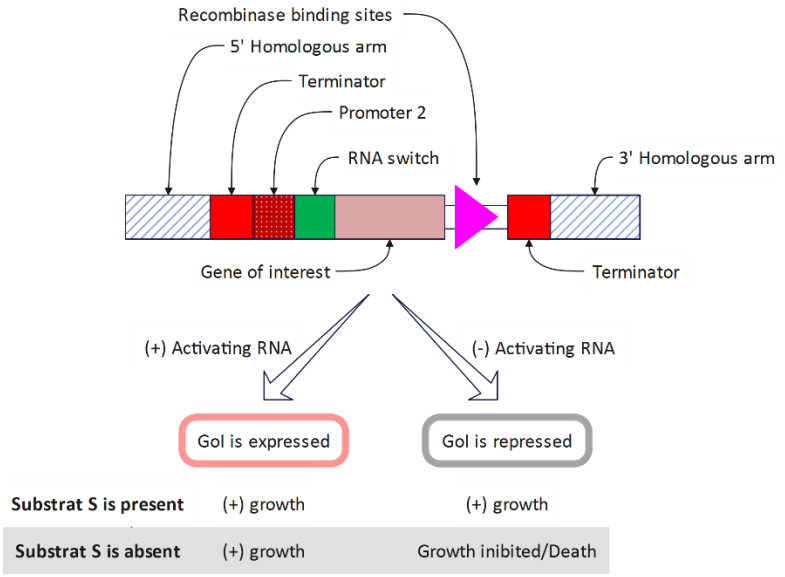
Figure 2: Chromosomally-integrated "Activating RNA-Inducible Gene of Interest" (ARIGoI) after Flp-mediated excision of the antibiotic resistance and SacB cassettes.

The promoter (promoter 1) that drives π or parA expression can be of varying strength. Their Ribosome binding site's sequence can be modified in order to modulate plasmid copy number (for detail, see Ref [34]). Further strategies for varying plasmid copy numbers are disclosed in the prior art [38,39].

The Gene of Interest (GoI) of an ARIGoI encodes for an "important polypeptide", required for synthesizing an essential substrate S. The GoI might correspond to *Escherichia coli's* argA, hisB, ilvA, leuB, lysA, metA, proA, pheA, thrC, trpC, or tyrA, where S corresponds to the amino acid L-arginine, L-Histidine, L-Isoleucine, L-Leucine, L-Lysine, L-Methionine, L-Proline, L-Phenylalanine, L-Threonine, L-Tryptophan, or tyrosine, respectively. Its promoter (promoter 2) is preferably devoid of a Ribosome binding site and can be selected from a collection of promoters of varying strength, such as Chris Anderson's promoters, iGEM's BIOFAB collection, or those disclosed in Ref [35,36].

The "important polypeptide" can comprise a degron sequence in its C-terminal. Exemplary degron sequences are disclosed in Table 1 of Ref [40]. An example of a popular degron is ssrA tag (AANDENYALAA) [41]. Without being bound to a particular theory or mechanism, lowering the polypeptide's half-life has the effect of improving the ON/OFF ratio (fold activation) of the system. This, in turn, leads to improved inhibition and/or elimination of bacteria lacking Petit Plasmids during selection.

A bacterial strain that supports the maintenance and replication of Petit Plasmids can be generated by first knocking-out a GoI using methods well known in the art [28,42,43], converting the cells into an auxotrophic strain for a substrate S, and then chromosomally integrating ARIGoI using methods well known in the art [44–46]. ARIGoI can comprise a replication cassette that encodes replication proteins required for the maintenance and replication of plasmids harboring an origin of replication, such as the minimal R6Kγ or the minimal colE2-P9. Alternatively, the replication cassette can be inserted during a distinct integration step. The resulting engineered conditionally-auxotrophic strain can be propagated in a medium supplemented with the substrate S or preserved by preparing glycerol stocks, which are stored at -80°C.



Substrat S is present	(+) growth	(+) growth
Substrat S is absent	(+) growth	Growth inibited/Death

Figure 3: Effects of the presence or absence of "activating RNAs" and "substrate S" on bacterial growth.

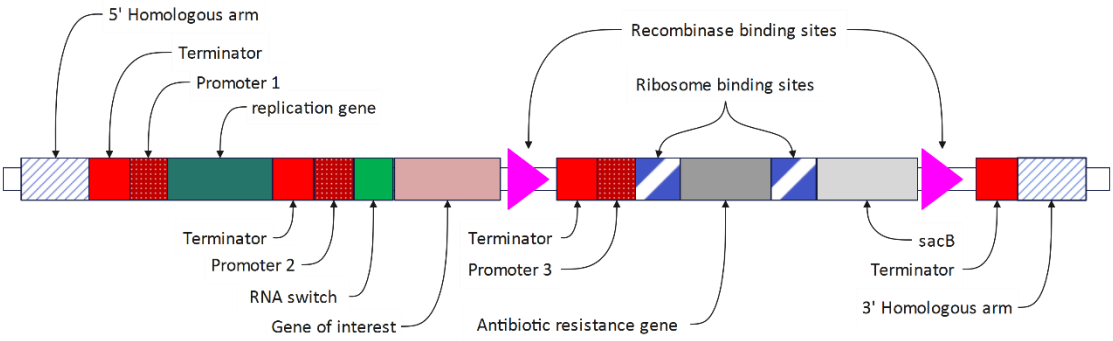


Figure 4: "Activating RNA-Inducible Gene of Interest" (ARIGoI) with an upstream replication cassette.

Following bacterial transformation with Petit Plasmids, transformants are selected by culturing cells in a chemically-defined agar plates or media that lacks the substrate S. Seed cultures can be started by streaking colonies on minimal agar plates lacking the substrate S. After incubation at 30-37°C for 11-18h, well-isolated and colony PCR-verified colonies are inoculated in media lacking the substrate S to provide 0.01-1% inoculums for use in laboratory-scale or large-scale fermentation. Media used for culture or fermentation should lack the substrate S, or should not be supplemented with the substrate S if it was initially present. Plasmids are extracted and purified using commercially-available kits or methods well known in the art [47].

Implementation

Lambda red recombineering is a useful tool for inserting a high molecular weight DNA sequence into specific target sites within a bacterial chromosome [44]. Here, I take advantage of this tool to simultaneously knock-out the lysA gene of *E. coli* and insert a cassette (referred to as pir_induc_lysA or parA_induc_lysA) comprising a replication cassette encoding a replication protein, a dual transcription-translation activator-controllable lysA gene, and an antibiotic resistance cassette. The replication protein can either be a π protein for the maintenance of a circular DNA plasmid bearing a minimal R6Kγ origin of replication or a repA protein for the maintenance of a circular DNA plasmid bearing a minimal colE2-P9 origin of replication. The antibiotic resistance cassette is removed by transforming the strain with a plasmid expressing an Flp recombinase [48].

DNA sequences and Plasmids

pR6Kγ MCS (pR6Kγ_MCS, sequence in Supplementary Table S2) is a circular DNA sequence comprising: an R6Kγ origin of replication, and a multiple cloning site (MCS).

Petit plasmid R6Kγ noPT (PP_R6Kγ_noPT_MCS, sequence in Supplementary Table S2) is a circular DNA sequence comprising: an R6Kγ origin of replication, a small transcription activating RNA (STAR), and a multiple cloning site (MCS).

Petit plasmid R6Kγ MCS (PP_R6Kγ_MCS, sequence in Supplementary Table S2) is a circular DNA sequence comprising: an R6Kγ origin of replication, a promoter, a small transcription activating RNA (STAR), a terminator, and a multiple cloning site (MCS).

Petit plasmid R6Kγ Tol2 (PP_R6Kγ_Tol2_MCS, a transposon plasmid, sequence in Supplementary Table S2) is a circular DNA sequence comprising: an R6Kγ origin of replication, a promoter, a small transcription activating RNA (STAR), a terminator, a truncated 5’ flanking region of a Tol2 transposon, a multiple cloning site (MCS), and a truncated 3’ flanking region of a Tol2 transposon.

Petit plasmid R6Kγ ePB (PP_R6Kγ_ePB_MCS, a transposon plasmid, sequence in Supplementary Table S2) is a circular DNA sequence comprising: an R6Kγ origin of replication, a promoter, a small transcription activating RNA (STAR), a terminator, a truncated 5’ flanking region of a Piggybac transposon, a multiple cloning site (MCS), and a truncated 3’ flanking region of a Piggybac transposon.

Petit plasmid R6Kγ SB (PP_R6Kγ_SB_MCS, a transposon plasmid, sequence in Supplementary Table S2) is a circular DNA sequence comprising: an R6Kγ origin of replication, a promoter, a small transcription activating RNA (STAR), a terminator, a truncated 5’ flanking region of a Sleeping Beauty transposon, a multiple cloning site (MCS), and a truncated 3’ flanking region of a Sleeping Beauty transposon.

Plasmid pR6Kγ_eGFP, PP_R6Kγ_eGFP_noPT, PP_R6Kγ_eGFP, PP_R6Kγ_eGFP_Tol2, PP_R6Kγ_eGFP_ePB, and PP_R6Kγ_eGFP_SB are circular DNA sequences obtained by cloning an enhanced Green Fluorescent Protein (eGFP) cassette into the MCS of pR6Kγ_MCS, PP_R6Kγ_noPT_MCS, PP_R6Kγ_MCS, PP_R6Kγ_Tol2_MCS, PP_R6Kγ_ePB_MCS, and PP_R6Kγ_SB_MCS, respectively. The sequence of eGFP cassette is in Supplementary Table S2.

pR6Kγ_Tol2_MCS (a transposon plasmid, sequence in Supplementary Table S2) is a circular DNA sequence comprising: an R6Kγ origin of replication, a truncated 5’ flanking region of a Tol2 transposon, a multiple cloning site (MCS), and a truncated 3’ flanking region of a Tol2 transposon.

pR6Kγ_Tol2_mCherry is a circular DNA sequence obtained by cloning a DsRed Fluorescent Protein (mCherry) cassette (sequence in Supplementary Table S2) into the MCS of pR6Kγ_Tol2_MCS. The mCherry cassette comprises: a CMV promoter, a *Homo sapiens* codon-optimized DsRed Fluorescent Protein gene, a small transcription activating RNA (STAR) cassette, and a polyadenylation sequence. The STAR cassette comprises: a promoter, a sequence encoding a small transcription activating RNA, and a terminator.

pcolE2-P9 MCS (pcolE2-P9_MCS, sequence in Supplementary Table S2) is a circular DNA sequence comprising: a colE2-P9 origin of replication, and a multiple cloning site (MCS).

Petit plasmid colE2-P9 noPT (PP_colE2-P9_noPT_MCS, sequence in Supplementary Table S2) is a circular DNA sequence comprising: a colE2-P9 origin of replication, a small transcription activating RNA (STAR), and a multiple cloning site (MCS).

Petit plasmid colE2-P9 MCS (PP_colE2-P9_MCS, sequence in Supplementary Table S2) is a circular DNA sequence comprising: a colE2-P9 origin of replication, a promoter, a small transcription activating RNA (STAR), a terminator, and a multiple cloning site (MCS).

Petit plasmid colE2-P9 Tol2 (PP_colE2-P9_Tol2_MCS, a transposon plasmid, sequence in Supplementary Table S2) is a circular DNA sequence comprising: a colE2-P9 origin of replication, a promoter, a small transcription activating RNA (STAR), a terminator, a truncated 5’ flanking region of a Tol2 transposon, a multiple cloning site (MCS), and a truncated 3’ flanking region of a Tol2 transposon.

Petit plasmid colE2-P9 ePB (PP_colE2-P9_ePB_MCS, a transposon plasmid, sequence in Supplementary Table S2) is a circular DNA sequence comprising: a colE2-P9 origin of replication, a promoter, a small transcription activating RNA (STAR), a terminator, a truncated 5’ flanking region of a Piggybac transposon, a multiple cloning site (MCS), and a truncated 3’ flanking region of a Piggybac transposon.

Petit plasmid colE2-P9 SB (PP_colE2-P9_SB_MCS, a transposon plasmid, sequence in Supplementary Table S2) is a circular DNA sequence comprising: a colE2-P9 origin of replication, a promoter, a small transcription activating RNA (STAR), a terminator, a truncated 5’ flanking region of a Sleeping Beauty transposon, a multiple cloning site (MCS), and a truncated 3’ flanking region of a Sleeping Beauty transposon.

Plasmid pcolE2-P9_eGFP, PP_colE2-P9_eGFP_noPT, PP_colE2-P9_eGFP, PP_colE2-P9_eGFP_Tol2, PP_colE2-P9_eGFP_ePB, and PP_colE2-P9_eGFP_SB are circular DNA sequences obtained by cloning an enhanced Green Fluorescent Protein (eGFP) cassette into the MCS of pcolE2-P9_MCS, PP_colE2-P9_noPT_MCS, PP_colE2-P9_MCS, PP_colE2-P9_Tol2_MCS, PP_colE2-P9_ePB_MCS, and PP_colE2-P9_SB_MCS, respectively. The sequence of eGFP cassette is in Supplementary Table S2.

pcolE2-P9_Tol2_MCS (a transposon plasmid, sequence in Supplementary Table S2) is a circular DNA sequence comprising: an colE2-P9 origin of replication, a truncated 5’ flanking region of a Tol2 transposon, a multiple cloning site (MCS), and a truncated 3’ flanking region of a Tol2 transposon.

pcolE2-P9_Tol2_mCherry is a circular DNA sequence obtained by cloning a DsRed Fluorescent Protein (mCherry) cassette (sequence in Supplementary Table S2) into the MCS of pcolE2-P9_Tol2_MCS. The mCherry cassette comprises: a CMV promoter, a *Homo sapiens* codon-optimized DsRed Fluorescent Protein gene, a small transcription activating RNA (STAR) cassette, and a polyadenylation sequence. The

STAR cassette comprises: a promoter, a sequence encoding a small transcription activating RNA, and a terminator.

The cassette parA_induc_lysA (sequence in Supplementary Table S2) is a linear DNA sequence comprising: a 79-nt left homologous arm taken from the 5' region of lysA gene, a terminator sequence to attenuate the transcriptional interference caused by the upstream lysA promoter, a constitutive promoter, a sequence encoding parA protein, a terminator, a constitutive promoter, a dual transcription-translation activator sequence, a sequence encoding *E. coli* diaminopimelate decarboxylase (lysA), a wild-type Flp recombinase binding site (Frt), a terminator, a constitutive promoter, a sequence encoding aminoglycoside phosphotransferase (kanR), a ribosome binding site, a sequence encoding *B. subtilis* Levansucrase (sacB), a wild-type Flp recombinase binding site, a bidirectional terminator, and an 80-nt right homologous arm taken from the 3' region of lysA gene.

The cassette pir_induc_lysA (sequence in Supplementary Table S2) is a linear DNA sequence comprising: a 79-nt left homologous arm taken from the 5' region of lysA gene, a terminator sequence to attenuate the transcriptional interference caused by the upstream lysA promoter, a constitutive promoter, a sequence encoding π protein, a terminator, a constitutive promoter, a dual transcription-translation activator sequence, a sequence encoding *E. coli* diaminopimelate decarboxylase (lysA), a wild-type Flp recombinase binding site (Frt), a terminator, a constitutive promoter, a sequence encoding aminoglycoside phosphotransferase (kanR), a ribosome binding site, a sequence encoding *B. subtilis* Levansucrase (sacB), a wild-type Flp recombinase binding site, a bidirectional terminator, and an 80-nt right homologous arm taken from the 3' region of lysA gene.

parA_induc_lysA and pir_induc_lysA will serve for the generation of engineered auxotrophic strains that support the maintenance and replication of circular DNA plasmids bearing a minimal colE2-P9 and R6Kγ origin of replication, respectively.

pir_induc_lysA or parA_induc_lysA can be synthesized de-novo in full or in fragments, which are combined together using cloning kits such as Master Mix Gibson assembly®. SacB is used for negative selection and is optional. Its coding sequence can be removed from the construct to reduce its size. KanR is used for positive selection in the presence of the antibiotic Kanamycin. KanR and SacB are excised from the bacterial chromosome by transforming the cell with a plasmid expressing Flp recombinases.

The cassette pir_induc_lysA or parA_induc_lysA is PCR-amplified with the following primers (designed from the sequence NZ_JANCYZ010000002, genbank):

- Forward: 5'-GCACTTATCTGGAGTTTGTTATGCCACATTC-3',
- And Reverse: 5'- GTCATCATGCAACCAGCGACTAACC -3'.

PCR products are run on agarose gel, then the band of the correct size is cut-out and processed using an appropriate gel extraction kit by following instructor's manual.

Strain engineering

Some exemplary *Escherichia coli* strains suitable for the generation of engineered auxotrophic strains are available in Table 1. If the strain is commercially-available, vendor's instructions should be followed carefully. For example, Stbl3® strains are already chemically competent and shouldn't be used for electroporation. After the completion of a given step, glycerol stocks of any generated strains can be prepared and then stored at the appropriate temperature (-80°C).

Here, I disclose a method of obtaining engineered L-lysine auxotrophic strains that can support the maintenance and replication of plasmids bearing an R6Kγ origin of replication. Adapting this method to generate engineered L-lysine auxotrophic strains that can support the maintenance and replication of plasmids bearing a colE2-P9 origin of replication is straightforward and consists of replacing pir_induc_lysA with parA_induc_lysA in the protocols.

The amounts of antibiotics added to culture media are: kanamycin (50 $\mu g.mL^{-1}$), ampicillin (100 $\mu g.mL^{-1}$), and chloramphenicol (34 $\mu g.mL^{-1}$).

Table 1: Escherichia coli strains

Name	Genotype
Stbl2®	F− endA1 glnV44 thi1 recA1 gyrA96 relA1 Δ(lac-proAB) mcrA Δ(mcrBC-hsdRMS-mrr) λ−
Stbl3®	F−mcrB mrr hsdS20 (rB−, mB−) recA13 supE44 ara14 galK2 lacY1 proA2 rpsL20 (StrR) xyl5 λ−leu mtl1
DH5α	F− φ80dlacZΔM15 Δ(lacZYA-argF)U169 endA1 recA1 hsdR17 (rK− mK+) deoR thi1 phoA supE44 λ−gyrA96 relA1
DH10B	F−mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 endA1 recA1 deoR Δ(ara, leu)7,697 araD139 galU galK nupG rpsL λ−
Top10	F−mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7,697 galU galK rpsL (StrR) endA1 nupG

pKD46 is a temperature-sensitive plasmid that encodes an arabinose-inducible lambda red recombination system. It is transfected into a selected strain by chemical transformation or electroporation. Successful recombinants are selected on plates with ampicillin at a temperature between 30-32 °C. After overnight growth, 5-20 colonies are inoculated into LB (Luria Bertani) medium with ampicillin and screened by colony PCR to identify transformants harboring the pKD46-encoded lambda red recombination system. Media containing positive colonies are grown to an OD_{600} of 0.2 at 30°C. 1% (w/v) of L-arabinose is added to the media, and the culture is grown at 30°C until the OD_{600} reaches 0.4-0.6. Before performing electroporation, the cells should be washed with ice-cold deionized water 2-3 times by repeated pelleting (by centrifugation) and resuspension. After the last pelleting, cells are resuspended in 10% glycerol.

The gel-purified linear pir_induc_lysA is electroporated or chemically transformed into the cells. Use between 30-150 µl of the cell culture and add a volume containing 2-5 µl of pir_induc_lysA DNA. After transformation, immediately pour the suspension into 1-2 mL of LB medium (Luria Bertani broth contains L-lysine) and allow the cells to recover by incubating at 37°C for 1 hour. Spread the bacterial culture on LB plates with kanamycin and incubate overnight at 37°C. Pick 5-20 colonies, inoculate into LB (Luria Bertani) medium with kanamycin, and screen by colony PCR to identify and confirm transformants harboring the chromosomally integrated pir_induc_lysA. The following primers are used for PCR-amplification (designed from the sequence NZ_JANCYZ010000002, genbank):

- Forward: 5'- GCTCTCTCGCAATCCGGTAATCC-3'
- Reverse: 5'- CATTCAGTGTCAACTCCGTCGC-3'

Bands of the correct size are gel-purified and sent for sequencing. Positive transformants will be transformed with the plasmid pCP20 to remove the antibiotic resistance and/or the sacB gene.

pCP20 is a temperature-sensitive plasmid that encodes a temperature-inducible Flp recombinase. It is transformed into the cells by chemical transformation or electroporation. After transformation, immediately pour the suspension into 1-2 mL of LB medium (Luria Bertani broth contains L-lysine) and allow the cells to recover by incubating at 30°C for 1 hour. Spread the bacterial culture on LB plates with chloramphenicol and incubate overnight at 30°C. Pick 5-20 colonies, inoculate into LB (Luria Bertani) medium with 6% sucrose (w/v), and incubate overnight at 42-43°C.

Prepare 10^4-10^6 dilutions of the overnight culture and plate 50 µL of the dilutions on LB plates. Incubate overnight at 30°C. Select randomly 5-20 colonies and patch on LB + chloramphenicol, LB + kanamycin, LB + ampicillin, and LB. Inoculate the colonies in 5 mL of LB medium. Incubate the plates and media at 37°C overnight. Select for colonies that are kanamycin, chloramphenicol, ampicillin sensitive, and that show growth on LB plates. PCR-verify the colonies to check for successful elimination of KanR and/or SacB by using the following primers:

- Forward: 5'- GCTCTCTCGCAATCCGGTAATCC-3'
- Reverse: 5'- CATTCAGTGTCAACTCCGTCGC-3'

Bands of the correct size are gel-purified and sent for sequencing. The resulting strain should be L-Lysine auxotroph and capable of maintaining and replicating a plasmid harboring a minimal R6Kγ origin of replication. If the plasmid further comprises a small transcription activating RNA (STAR) sequence under the control of a constitutive promoter, diaminopimelate decarboxylase should be produced, and the transformed strain should be able to grow in the absence of L-Lysine.

Plasmids' maintenance, replication, extraction, and purification

An engineered *Escherichia coli* strain obtained by following the instructions herein is called *E. coli* ΔlysA::[π-dtta^{ind}(lysA)] or *E. coli* ΔlysA::[parA-dtta^{ind}(lysA)]. π: the strain constitutively expresses π protein. parA: the strain constitutively expresses parA protein. dtta^{ind}(lysA): the gene that encodes Diaminopimelate decarboxylase is under the control of a small transcription activating RNAs (STAR)-**ind**ucible dual transcription-translation activator sequence. Examples of strains are *E. coli* Stbl3® ΔlysA::[π-dtta^{ind}(lysA)] and *E. coli* DH10B ΔlysA::[parA-dtta^{ind}(lysA)].

The engineered strains are cultured on agar plates or in media containing and/or supplemented with L-lysine for their maintenance. Before transformation, cells are preferably rendered competent by following well-established protocols [49–52]. After their transformation with a given plasmid, cells can be cultured in 1-2 mL culture medium lacking or containing L-lysine at 30°C for 30 minutes to 1 hour to permit recovery. The culture can be spread on multiple minimal agar plates, such as M9 minimal agar plates without L-lysine and incubated at 37°C for 18-24 h to select for transformants. Positive colonies identified by colony-PCR can be cultured in minimal medium without L-lysine such as M9 minimal medium supplemented with glucose. 5-10 mL of culture medium can be used for standard molecular biology applications. Plasmids can be extracted and purified with commercially-available Miniprep kits by following the vendor's instructions.

The following plasmids are used for chemical transformation into engineered *E. coli* Stbl3® ΔlysA::[π-dtta^{ind}(lysA)] strains: pR6Kγ_eGFP, PP_R6Kγ_eGFP_noPT, PP_R6Kγ_eGFP, PP_R6Kγ_eGFP_Tol2, PP_R6Kγ_eGFP_ePB, PP_R6Kγ_eGFP_SB, and pR6Kγ_Tol2_mCherry. The following plasmids are used for chemical transformation into engineered *E. coli* Stbl3® ΔlysA::[parA-dtta^{ind}(lysA)]: pcolE2-P9_eGFP, PP_colE2-P9_eGFP_noPT, PP_colE2-P9_eGFP, PP_colE2-P9_eGFP_Tol2, PP_colE2-P9_eGFP_ePB, PP_colE2-P9_eGFP_SB, and pcolE2-P9_Tol2_mCherry. The parental strain *E. coli* Stbl3® is recommended

for the maintenance and replication of unstable plasmids containing direct repeats. Chemically transformed cells are added to 1.5 mL of Luria Bertani (LB) medium and incubated at 30°C for 1 h. The culture is spread on multiple M9 minimum agar plates supplemented with glucose and incubated at 37°C for 18-24 h. 5-20 colonies are screened by colony PCR and inoculated into 5 mL of M9 minimum media supplemented with glucose. Positive transformants are retained, and the culture media are incubated at 37°C for 12-16 h. Extract and purify DNA plasmids with commercially-available kits, such as Monarch® Plasmid Miniprep Kit by following the vendor's instructions.

Mammalian cells' transfection

Purified plasmids pR6Kγ_Tol2_mCherry and pcolE2-P9_Tol2_mCherry are used for mammalian cells' transfection. The goal is to integrate the Tol2_mCherry cassette into the genome of the transfected cells. Total and fluorescing cells are counted at regular intervals to assess integrant stability and determine transfection and integration efficiency. 3.10^5-5.10^6 HT1080 cells are transfected with a combination of plasmid pR6Kγ_Tol2_mCherry and pCAGGS-mT2TP (a Tol2 transposase-expressing vector) or pcolE2-P9_Tol2_mCherry and pCAGGS-mT2TP at 1-5 µg each, using FuGENE® 6 transfection reagent by following the vendor's instructions. Cells are cultured for 2 days, then trypsinized, counted, and sorted with a Fluorescense Activated Cell Sorting (FACS) system. Isolated fluorescing cells are further cultured for 2 days, trypsinized, counted, and FACS-sorted. This operation can be repeated during the next 6-10 days.

Expectation

Bacterial cells transformed with pR6Kγ_eGFP, PP_R6Kγ_eGFP_noPT, pcolE2-P9_eGFP, and PP_colE2-P9_eGFP_noPT will not yield any colonies harboring the plasmids after selection on M9 minimal agar plates lacking L-lysine. The absence of a STAR cassette, or the presence of sequence encoding STAR but without a promoter to drive its expression, renders the plasmids non-maintainable.

Bacterial cells transformed with PP_R6Kγ_eGFP, PP_R6Kγ_eGFP_Tol2, PP_R6Kγ_eGFP_ePB, PP_R6Kγ_eGFP_SB, pR6Kγ_Tol2_mCherry, PP_colE2-P9_eGFP, PP_colE2-P9_eGFP_Tol2, PP_colE2-P9_eGFP_ePB, PP_colE2-P9_eGFP_SB, and pcolE2-P9_Tol2_mCherry will yield colonies harboring the respective plasmids after selection on M9 minimal agar plates lacking L-lysine.

The mCherry cassette of the plasmids pR6Kγ_Tol2_mCherry and pcolE2-P9_Tol2_mCherry will be integrated into the genome of HT1080 cells. Tol2 transposases mediate the random genomic integration of the mCherry cassette, which is flanked by two minimal regions of Tol2 transposon that are necessary and sufficient for transposition [53,54].

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Conflict of interest statement

The author declares no conflict of interest.

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Supplementary materials

Table S1: DNA sequences of important parts

Name	Description	Sequence
R6Kγ_ori	R6Kγ origin of replication	TGTCAGCCGTTAAGTGTTCCTGTGTCACTCAAAATTGCTTTG AGAGGCTCTAAGGGCTTCTCAGTGCGTTACATCCCTGGCTT GTTGTCCACAACCGTTAAACCTTAAAAGCTTTAAAAGCCTTA TATATTCTTTTTTTTCTTATAAAACTTAAAACCTTAGAGGCTA TTTAAGTTGCTGATTTATATTAATTTTATTGTTCAAACATGA GAGCTTAGTACGTGAAACATGAGAGCTTAGTACGTTAGCC ATGAGAGCTTAGTACGTTAGCCATGAGGGTTTAGTTCGTTA AACATGAGAGCTTAGTACGTTAAACATGAGAGCTTAGTAC GTGAAACATGAGAGCTTAGTACGTAC
colE2-P9_ori	Minimal ColE2 origin	GAGCGCCTCAGCGCGCCGTAGCGTCGATAAAAATTACGGG CTGGGGCGAAACTACCATCTGTTCGAAAAGGTCCGTAAAT GGGCCTACAGAGCGATTCGTCAGGGCTGGCCTGTATTCTCA CAATGGCTTGATGCCGTTATCCAGCGTGTCGAAATGTACAA CGCTTCGCTTCCCGTTCCGCTTTCTCCGGCTGAATGTCGGGC TATTGGCAAGAGCATTGCGAAATATACACACAGGAAATTCT CACCAGAGGGATTTTCCGCTGTACAGGCCGCTCGCGGTCGC AAGGGCGGAACTAAATCTAAGCGCGCAGCAGTTCCTACAT CAGCACGTTCGCTGAAACCGTGGGAGGCATTAGGCATCAG TCGAGCGACGTACTACCGAAAATTAAAATGTGACCCAGACC TCGCAAAATGAGACCAGATAAGCCTTATCAGATAACAGCG CCCTTTTGGCGTCTTTTTGAGCA
Term_istr1	ISTR-1 terminator	CGAAACCTCGCTCCGGCGGGGTTTTTTGTTATCTGCA
Term_ BBa_B1005	BBa_B1005 terminator	CGCCGCAAACCCCGCTTCGGCGGGGTTTCGCCGC
Term_bidir	Bidirectional terminator	AAATATAATGACCCTCTTGATAACCCAAGAGGGCATTTTTT A
Term_T7	T7 terminator	CTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGG GTTTTTTG
Term_T500	T500 terminator	CAAAGCCCGCCGAAAGGCGGGCTTTTTTTT
Term_1	Terminator	TCATGTTTGACAGCTTATCATCGAATTTCTGCCATTCATCCG CTTATTATCACTTATTCAGGCGTAGCAACCAGGCGTTTAAG GGCACCAATAACTGCCTTAAAAAAA
Term_2	Terminator	GGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGC CTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAG TAGGACAAATCCGCCGCCCTAGA
Tol2_5_prime	5’ flanking region of Tol2 transposon	CAGAGGTGTAAAGTACTTGAGTAATTTTACTTGATTACTGT ACTTAAGTATTATTTTTGGGGATTTTTACTTTACTTGAGTAC AATTAAAAATCAATACTTTTACTTTTACTTAATTACATTTTTT TAGAAAAAAAAGTACTTTTTACTCCTTACAATTTTATTTACA GTCAAAAAGTACTTATTTTTTGGAGATCACTT
Tol2_3_prime	3’ flanking region of Tol2 transposon	TAATACTCAAGTACAATTTTAATGGAGTACTTTTTTACTTTTA CTCAAGTAAGATTCTAGCCAGATACTTTTACTTTTAATTGAG TAAAATTTTCCCTAAGTACTTGTACTTTCACTTGAGTAAAAT TTTTGAGTACTTTTTACACCTCTG

SB_5_prime	5’ flanking region of Sleeping Beauty transposon	CAGTTGAAGTCGGAAGTTTACATACACTTAAGTTGGAGTCA TTAAAACTCGTTTTTCAACTACTCCACAAATTTCTTGTTAACA AACAATAGTTTTGGCAAGTCAGTTAGGACATCTACTTTGTG CATGACACAAGTCATTTTTCCAACAATTGTTTACAGACAGAT TATTTCACTTATAATTCACTGTATCACAATTCCAGTGGGTCA GAAGTTTACATACACTAAGT
SB_3_prime	3’ flanking region of Sleeping Beauty transposon	ATTGAGTGTATGTAAACTTCTGACCCACTGGGAATGTGATG AAAGAAATAAAAGCTGAAATGAATCATTCTCTCTACTATTA TTCTGATATTTCACATTCTTAAAATAAAGTGGTGATCCTAAC TGACCTAAGACAGGGAATTTTTACTAGGATTAAATGTCAGG AATTGTGAAAAAGTGAGTTTAAATGTATTTGGCTAAGGTGT ATGTAAACTTCCGACTTCAACTG
ePB_5_prime	5’ flanking region of enhanced piggyback transposon	CGCAGCTAGATTAACCCTAGAAAGATAGTCTGCGTAAAATT GACGCATGCATTCTTGAAATATTGCTCTCTCTTTCTAAATAG CGCGAATCCGTCGCTGTGCATTTAGGACATCTCAGTCGCCG CTTGGAGCTCCCGTGAGGCGTGCTTGTCAATGCGGTAAGT GTCACTGATTTTGAACTATAACAACCGCGTGAGTCAAAATG ACGCATGATTATCTTTTACGTGACTTTTAAGATTTAACTCAT ACGATAATTATATTGTTATTTCGTGTTCTACTTACGTGATAA CTTATTATATATATATTTTCTTGTTATAGATATCCTT
ePB_3_prime	3’ flanking region of enhanced piggyback transposon	CGATAAAAGTTTTGTTACTTTATAGAAGAAATTTTGAGTTTT TGTTTTTTTTTAATAAATAAATAAACATAAATAAATTGTTTG TTGAATTTATTATTAGTATGTAAGTGTAAATATAATAAAACT TAATATCTATTCAAATTAATAAATAAACCTCGATATACAGAC CGATAAAACACATGCGTCAATTTTACGCATGATTATCTTTAA CGTACGTCACAATATGATTATCTTTCTAGGGTTAATCTAGCT GC
HA_left_lysA	Left homologous arm from the 5’ region of *E. coli* lysA gene	GCACTTATCTGGAGTTTGTTATGCCACATTCACTGTTCAGCA CCGATACCGATCTCACCGCCGAAAATCTGCTGCGTTT
HA_right_lysA	Right homologous arm from the 3’ region of *E. coli* lysA gene	ATTCGCCGTCGCCAGACCATCGAAGAATTACTGGCGCTGGA ATTGCTTTAACTGCGGTTAGTCGCTGGTTGCATGATGAC
Pir_seq	Sequence encoding π protein	ATGCGCCTGAAAGTGATGATGGATGTGAACAAGAAGACCA AGATTCGCCATCGCAACGAACTGAACCATACCCTGGCGCAG CTGCCGTTACCGGCGAAACGCGTGATGTATATGGCGCTGG CACCGATTGATAGCAAAGAACCGCTGGAACGCGGCCGCGT GTTTAAGATTCGCGCGGAAGATCTGGCAGCGCTGGCGAAG ATTACGCCGAGCCTGGCATATCGCCAGCTGAAAGAAGGCG GCAAGCTGCTGGGTGCGAGCAAGATTAGCCTGCGCGGCGA TGATATTATTGCGCTGGCGAAAGAACTGAACCTGCCGTTTA CCGCGAAGAACTCTCCGGAAGAACTGGATCTGAACATTATT GAATGGATTGCGTATAGCCCGGATGAAGGCTATCTGAGCC TGAAATTTACCCGCACCATTGAACCGTATATCAGCAGCCTG ATTGGCAAGAAGAACAAATTTACCACCCAGCTGCTGACCGC GAGCCTGCGCCTGAGCTCTCAGTATAGTAGCAGCCTGTATC AGCTGATTCGCAAACACTACAGCAACTTTAAGAAGAAGAA CTACTTCATCATCAGCGTGGATGAGCTGAAAGAAGAACTG

		ATTGCGTATACCTTTGACAAGGACGGCAACATTGAATATAA ATACCCGGATTTCCCGATCTTTAAACGCGATGTGCTGAACA AAGCGATTGCGGAAATTAAGAAGAAGACCGAAATTAGCTT TGTGGGCTTTACCGTGCATGAGAAAGAAGGCCGCAAGATT AGCAAACTGAAATTTGAATTTGTGGTGGATGAGGACGAAT TCAGCGGCGATAAAGATGATGAAGCGTTCTTTATGAACCTG AGCGAAGCGGATGCGGCGTTCCTGAAAGTGTTTGATGAAA CCGTGCCGCCGAAGAAAGCGAAAGGCTAA
parA_seq	Sequence encoding parA protein	ATGAGCGCGGTGCTGCAGCGCTTTCGCGAGAAGCTGCCGC ATAAACCGTATTGCACCAACGATTTCGCGTATGGCGTGCGC ATTCTGCCGAAGAACATTGCGATTCTGGCGCGCTTTATTCA GCAGAACCAGCCGCATGCGCTGTATTGGCTGCCGTTTGATG TGGATCGCACCGGCGCGAGCATTGATTGGAGCGATCGCAA CTGCCCAGCACCGAACATTACCGTGAAGAATCCGCGCAAC GGCCATGCGCATCTGCTGTATGCGCTGGCGTTACCAGTTCG CACCGCACCGGATGCGTCTGCGTCTGCATTACGCTATGCGG CAGCGATTGAACGCGCGCTGTGCGAGAAACTGGGTGCGGA TGTGAACTATAGCGGCCTGATTTGCAAGAATCCGTGCCATC CGGAATGGCAGGAAGTGGAATGGCGCGAAGAGCCGTATA CCCTGGATGAACTGGCGGATTATCTGGATCTGAGCGCGAG CGCGCGCCGTAGCGTGGATAAGAACTATGGCTTAGGCCGC AACTATCATCTGTTTGAGAAAGTGCGCAAATGGGCGTATCG CGCGATTCGCCAGGGCTGGCCGGTATTTAGCCAATGGCTG GATGCGGTGATTCAGCGCGTGGAAATGTATAACGCGAGCC TGCCGGTGCCGTTGAGCCCAGCAGAATGCCGTGCAATTGG TAAGAGCATTGCGAAATATACCCATCGCAAATTTAGTCCGG AAGGCTTCAGCGCGGTGCAGGCAGCACGTGGTCGTAAAGG TGGTACCAAATCTAAACGCGCGGCGGTGCCGACCAGCGCA CGTTCTTTAAAGCCATGGGAAGCACTCGGCATTAGCCGCGC GACCTATTATCGCAAACTGAAATGCGATCCGGATCTGGCGA AATAA
lysA_seq	Sequence encoding *Escherichia coli* Diaminopimelate decarboxylase	ATGCCGCATAGCCTGTTTAGCACCGATACCGATCTGACCGC GGAGAACCTGCTGCGCCTGCCAGCAGAATTTGGTTGTCCG GTGTGGGTGTATGATGCGCAGATTATTCGCCGCCAGATTGC GGCGCTGAAACAGTTTGATGTGGTGCGCTTTGCGCAGAAA GCGTGCAGCAACATTCATATTCTGCGCCTGATGCGCGAACA GGGCGTGAAAGTGGATAGCGTGAGCCTGGGTGAAATTGA ACGCGCGCTGGCGGCGGGCTATAACCCACAGACCCATCCG GATGATATTGTGTTTACCGCGGATGTGATTGATCAGGCGAC CCTGGAACGCGTGAGCGAGCTGCAGATTCCGGTGAATGCA GGCAGCGTGGATATGCTGGATCAGCTGGGCCAGGTGAGCC CAGGTCATCGCGTGTGGTTACGTGTGAACCCAGGCTTTGGC CATGGCCATAGCCAGAAGACCAACACCGGCGGCGAGAACA GCAAACATGGCATTTGGTATACCGATCTGCCGGCGGCGCT GGATGTGATTCAGCGCCATCATCTGCAGCTGGTTGGCATTC ATATGCATATTGGCAGCGGCGTGGATTATGCGCATCTGGA ACAGGTGTGCGGCGCGATGGTGCGTCAGGTGATTGAATTT GGCCAGGATCTGCAGGCGATTAGCGCGGGCGGCGGCTTAA

		GCGTTCCATATCAGCAGGGTGAAGAAGCGGTGGATACCGA ACATTATTATGGCCTGTGGAACGCGGCGCGCGAACAGATT GCGCGTCATTTGGGTCATCCGGTGAAACTGGAAATTGAACC GGGCCGCTTCCTGGTGGCGCAGAGCGGCGTTCTTATTACCC AAGTTCGCAGCGTGAAACAGATGGGCAGCCGCCATTTCGT GCTGGTGGATGCGGGCTTTAACGATCTGATGCGCCCGGCG ATGTATGGCAGCTATCATCATATTAGCGCGCTGGCGGCGG ATGGCCGCAGCTTAGAACATGCGCCAACTGTTGAAACCGT GGTGGCGGGCCCGCTGTGCGAATCTGGTGATGTGTTTACC CAGCAGGAAGGCGGCAACGTGGAAACCCGCGCGCTGCCA GAAGTTAAAGCAGGCGATTATCTGGTGCTGCATGATACCG GCGCGTATGGCGCGAGCATGAGCAGCAACTATAACAGCCG CCCACTGCTGCCGGAAGTGCTGTTTGATAACGGCCAGGCAC GCTTAATTCGCCGCCGCCAGACCATTGAAGAACTGCTGGCG CTGGAACTGCTGTGA
KanR_seq	Sequence encoding aminoglycoside phosphotransferase	ATGAGCCATATTCAGCGCGAAACCAGCTGCAGCCGCCCGC GCCTGAACTCTAATATGGATGCGGATCTGTATGGCTATAAA TGGGCGCGCGATAACGTGGGCCAGAGCGGCGCGACCATTT ATCGCTTATATGGCAAACCGGATGCGCCGGAACTGTTTCTG AAACATGGCAAAGGCAGCGTGGCGAACGATGTGACCGAT GAAATGGTGCGCCTGAACTGGCTGACCGAATTTATGCCGCT GCCGACCATTAAACATTTCATTCGCACGCCGGATGATGCGT GGCTGCTGACCACCGCGATTCCAGGTAAGACCGCATTTCAG GTGCTGGAAGAATATCCGGATAGCGGCGAGAACATTGTGG ATGCGCTGGCGGTGTTTCTGCGCCGCCTGCATAGCATTCCG GTGTGCAACTGCCCGTTTAACAGCGATCGCGTGTTTCGCCT GGCGCAGGCGCAGAGTCGTATGAACAACGGCCTGGTGGAT GCAAGCGATTTCGATGATGAACGCAACGGCTGGCCGGTGG AACAGGTGTGGAAGGAAATGCATAAGCTGCTGCCGTTCAG CCCGGATAGCGTGGTGACCCATGGTGATTTCAGCCTGGATA ACCTGATCTTCGATGAAGGCAAACTGATTGGCTGCATTGAT GTGGGCCGCGTTGGCATTGCGGATCGCTATCAGGATCTGG CGATTCTGTGGAACTGCCTGGGCGAATTTAGCCCGAGCCTG CAGAAACGCCTGTTTCAGAAGTACGGCATTGACAATCCGG ACATGAACAAACTGCAGTTTCATCTGATGCTGGATGAATTC TTTTAA
SacB_seq	Sequence encoding *Bacillus subtilis* Levansucrase	ATGAACATTAAGAAATTTGCGAAGCAGGCGACCGTGCTGA CCTTTACCACCGCGCTGTTAGCAGGTGGCGCAACTCAGGCG TTTGCGAAAGAGACCAACCAGAAACCGTATAAAGAAACCT ATGGCATTAGCCATATTACCCGCCATGATATGCTGCAGATT CCGGAACAGCAGAAGAACGAGAAATATCAGGTGCCGGAA TTTGATAGCAGCACCATTAAGAACATTAGCAGCGCGAAAG GCCTGGATGTGTGGGATAGCTGGCCGCTGCAGAACGCAGA TGGCACCGTGGCGAACTATCATGGCTATCATATTGTGTTTG CGCTGGCGGGCGATCCGAAGAACGCGGATGATACCAGCAT TTATATGTTCTATCAGAAGGTGGGCGAAACCAGCATTGATA GCTGGAAGAACGCCGGCCGCGTGTTTAAAGATAGCGATAA ATTTGATGCGAACGATAGCATTCTGAAGGACCAGACCCAG

		GAATGGAGCGGCAGCGCGACCTTTACCAGCGATGGCAAGA TTCGCCTGTTCTATACCGATTTCAGCGGCAAACATTATGGCA AACAGACCCTGACCACCGCGCAGGTGAACGTGAGCGCGTC TGATAGCAGCCTGAACATTAACGGCGTGGAAGATTATAAA TCGATCTTCGATGGCGATGGCAAGACCTATCAGAACGTGCA GCAGTTTATTGATGAGGGCAACTATAGCAGCGGCGATAAC CATACCCTGCGCGATCCGCATTATGTGGAAGATAAGGGCC ATAAATATCTGGTGTTTGAAGCGAACACCGGCACCGAAGA TGGCTATCAGGGCGAGGAGAGCCTGTTTAACAAAGCGTAT TATGGCAAATCTACCAGCTTCTTCCGCCAGGAAAGCCAGAA ACTGCTGCAGAGCGATAAGAAACGCACCGCGGAACTGGCG AACGGCGCACTGGGCATGATTGAACTGAACGATGACTACA CCCTGAAGAAAGTGATGAAACCGCTGATTGCGAGCAACAC CGTGACCGATGAAATTGAACGCGCGAACGTGTTTAAGATG AACGGCAAATGGTATCTGTTTACCGATAGCCGCGGCAGCA AGATGACCATTGATGGCATTACCAGCAACGATATTTATATG CTGGGCTATGTGAGCAACAGCCTGACCGGCCCGTATAAAC CGCTGAACAAGACCGGCCTGGTGCTGAAGATGGATCTGGA TCCGAACGATGTGACCTTTACCTATAGCCATTTCGCGGTGC CGCAGGCGAAGGGCAACAACGTGGTGATTACCAGCTATAT GACCAACCGCGGCTTCTATGCGGATAAACAGAGCACCTTTG CGCCGAGCTTCCTGCTGAACATTAAAGGCAAGAAGACCAG CGTGGTGAAAGATAGCATTCTGGAACAGGGCCAGCTGACC GTGAACAAATGA
eGFP_seq_ec	Sequence encoding an *E. coli* codon-optimized enhanced Green Fluorescent Protein	ATGGTGAGCAAAGGCGAAGAACTGTTTACCGGCGTGGTGC CGATTCTGGTGGAACTGGATGGCGATGTGAACGGCCATAA ATTTAGCGTGAGCGGCGAAGGCGAAGGCGATGCGACCTAT GGCAAACTGACCCTGAAATTTATTTGCACCACCGGCAAACT GCCGGTGCCGTGGCCGACCCTGGTGACTACTTTAACCTATG GCGTGCAGTGCTTTAGCCGCTATCCGGATCATATGAAACAG CATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCA GGAACGCACCATCTTCTTTAAAGATGACGGCAACTATAAGA CCCGCGCGGAAGTGAAATTTGAAGGCGATACCCTGGTGAA CCGCATTGAACTGAAAGGCATTGACTTTAAAGAAGACGGC AACATTCTGGGCCATAAACTGGAATACAACTACAACAGCCA TAACGTGTATATTATGGCGGATAAGCAGAAGAACGGCATT AAAGTGAACTTCAAGATCCGCCATAACATTGAAGATGGCA GCGTGCAGCTGGCGGATCATTATCAGCAGAACACGCCGAT TGGCGATGGCCCGGTGCTGCTGCCGGATAACCATTATCTGA GCACCCAGAGCGCGCTGAGCAAAGATCCGAACGAGAAAC GCGATCATATGGTGCTGCTGGAATTTGTGACCGCGGCGGG CATTACCTTAGGCATGGATGAACTGTATAAATAA
mCherry_seq_hs	5’UTR + Kozak consensus sequence + *Homo sapiens* codon-optimized DsRed Fluorescent protein + 3’UTR	ATCGCTTCAGTCAAGATTTAATTAACGCCGCCACCATGGTG AGCAAGGGCGAGGAGGACAACATGGCCATCATCAAGGAG TTCATGAGGTTCAAGGTGCACATGGAGGGCAGCGTGAACG GCCACGAGTTCGAGATCGAGGGAGAGGGAGAAGGAAGGC CCTACGAGGGCACCCAGACAGCCAAGTTGAAGGTGACCAA GGGCGGACCCTTGCCCTTCGCCTGGGACATCCTTTCTCCTC

		AGTTCATGTACGGCAGCAAGGCCTACGTGAAGCACCCTGC CGACATCCCTGACTACCTGAAGCTGAGCTTCCCTGAGGGCT TCAAGTGGGAGAGGGTGATGAACTTCGAGGACGGCGGCG TGGTGACCGTGACACAGGATTCTAGCCTTCAGGATGGCGA GTTCATCTACAAGGTGAAGCTGAGAGGCACCAACTTCCCTA GCGACGGCCCTGTGATGCAGAAGAAGACCATGGGCTGGG AGGCCAGCTCTGAGAGGATGTACCCTGAGGACGGAGCTCT GAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGG CGGCCACTACGACGCCGAGGTGAAGACCACATACAAGGCC AAGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTGAACA TCAAGCTGGACATCACCAGCCACAACGAGGACTACACCATC GTGGAGCAGTACGAGAGGGCCGAGGGCAGGCATAGCACA GGAGGAATGGACGAGCTGTACAAGTGATAAAGCCTCAATA AAGCTTGCCTCGACCAG
polyA_BGH	Bovine Growth Hormone Polyadenylation sequence	CTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCC CCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTC CTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAG TAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGAC AGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCT GGGGATGCGGTGGGCTCTATGG
Prom_kanR	Promoter that drives KanR expression	AAAGCCACGTTGTGTCTCAAAATCTCTGATGTTACATTGCAC AAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCT TACATAAACAGTAATACAAGGGGTGTT
Prom_ BBa_J23108	Anderson promoter (BBa_J23108)	CTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGC
Prom_ BBa_J23105	Anderson promoter (BBa_J23105)	TTTACGGCTAGCTCAGTCCTAGGTACTATGCTAGC
Prom_ BBa_J23104	Anderson promoter (BBa_J23104)	TTGACAGCTAGCTCAGTCCTAGGTATTGTGCTAGC
Prom_1	Promoter	CACAGCTAACACCACGTCGTCCCTATCTGCTGCCCTAGGTCT ATGAGTGGTTGCTGGATAACTTTACGGGCATGCATAAGGCT CGTAGGCTATATTCAGGGAGACCACAACGGTTTCCCTCTAC AAATAATTTTGTTTAACTTT
Prom_CMV	Cytomegalovirus immediate early enhancer and promoter	TAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATA GCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAAT GGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGAC GTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGA CTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACT GCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTAC GCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGC ATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGC AGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGC GGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGA CTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATG GGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAA TGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAG GCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTTA GTGAACCGTCAGATC

RBS_ rhaS_20k	Native rhaS Ribosome Binding Site 20k	TATTTCGCCGTGTTGACGACATCAGGAGGCCAGT
RBS_1	Ribosome binding site	GAAATAAGGAGGTAATACAA
RBS_ BBa_K143021	Ribosome binding site	TACTAGAGAAAGGTGGTGAATACTAG
Rec_frt_wt	Flp recombinase binding site (wild-type)	GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC
Codon_stp_mf	Multiframe STOP codon	TTAGTTAGTTAG
LysA_HA_lft	lysA gene, left homology arm	GCACTTATCTGGAGTTTGTTATGCCACATTCACTGTTCAGCA CCGATACCGATCTCACCGCCGAAAATCTGCTGCGTTT
LysA_HA_rgt	lysA gene, right homology arm	ATTCGCCGTCGCCAGACCATCGAAGAATTACTGGCGCTGGA ATTGCTTTAACTGCGGTTAGTCGCTGGTTGCATGATGAC
Tta1_pT181	Dual transcription-translation activator vers.1 derived from pT181	TTGGGTGAGCGATTCCTTAAACGAAATTGAGATTAAGGAG TCGATTTTTTATGTATAAAAAC
Tta2_pT181	Dual transcription-translation activator vers.2 derived from pT181	TTGGGTGAGCGATTCCTTAAACGAAATTGAGATTAAGGAG TCGATTTTTT
Tta1_ pT181_eng	Engineered dual transcription-translation activator vers.1	TTGGGTGAGCGATTCCTTAAACGAAATTGAGATTAAGGAG TCGCTCTTTTTTTTTTATGTATAAAAAC
Tta2_ pT181_eng	Engineered dual transcription-translation activator vers.2	TTGGGTGAGCGATTCCTTAAACGAAATTGAGATTAAGGAG TCGCTCTTTTTTTTTT
STAR_1	Small transcription activating RNA (STAR)	AACAAAATAAAGCAATAAGGAATCGCTCACCCAAAGGATC T
MCS	Multiple cloning sites	ACGCGTCGCGAGGCCATATGGGTTAACCCATGGCCAAGCT TGCATGCCTGCAGGTCGACTCTAGAGGATCCCGGGTACCG AGCTCGAATTCGGATATCCTCGAGACTAGTGGGCCCGTTTA AACACATG

Table S2: Constructs' architecture

Construct name	Sequence architecture (5’ -> 3’)
pR6Kγ_MCS	R6Kγ_ori; MCS
PP_R6Kγ_noPT_MCS	R6Kγ_ori; STAR_1; MCS
PP_R6Kγ_MCS	R6Kγ_ori; Prom_BBa_J23105; STAR_1; Term_T500; MCS
PP_R6Kγ_Tol2_MCS	R6Kγ_ori; Prom_BBa_J23105; STAR_1; Term_T500; Tol2_5_prime; MCS; Tol2_3_prime
PP_R6Kγ_ePB_MCS	R6Kγ_ori; Prom_BBa_J23105; STAR_1; Term_T500; ePB_5_prime; MCS; ePB_3_prime
PP_R6Kγ_SB_MCS	R6Kγ_ori; Prom_BBa_J23105; STAR_1; Term_T500; SB_5_prime; MCS; SB_3_prime
eGFP_cassette	Prom_BBa_J23104; RBS_ BBa_K143021; eGFP_seq_ec; Term_2

pR6Kγ_eGFP	R6Kγ_ori; eGFP_cassette
PP_R6Kγ_eGFP_noPT	R6Kγ_ori; STAR_1; eGFP_cassette
PP_R6Kγ_eGFP	R6Kγ_ori; Prom_BBa_J23105; STAR_1; Term_T500; eGFP_cassette
PP_R6Kγ_eGFP_Tol2	R6Kγ_ori; Prom_BBa_J23105; STAR_1; Term_T500; Tol2_5_prime; eGFP_cassette; Tol2_3_prime
PP_R6Kγ_eGFP_ePB	R6Kγ_ori; Prom_BBa_J23105; STAR_1; Term_T500; ePB_5_prime; eGFP_cassette; ePB_3_prime
PP_R6Kγ_eGFP_SB	R6Kγ_ori; Prom_BBa_J23105; STAR_1; Term_T500; SB_5_prime; eGFP_cassette; SB_3_prime
STAR_cassette	Prom_BBa_J23105; STAR_1; Term_T500
mCherry_cassette	Prom_CMV; mCherry_seq_hs; STAR_cassette; polyA_BGH
pR6Kγ_Tol2_MCS	R6Kγ_ori; Tol2_5_prime; MCS; Tol2_3_prime
pR6Kγ_Tol2_mCherry	R6Kγ_ori; Tol2_5_prime; mCherry_cassette; Tol2_3_prime
pcolE2-P9_MCS	colE2-P9_ori; MCS
PP_colE2-P9_noPT_MCS	colE2-P9_ori; STAR_1; MCS
PP_colE2-P9_MCS	colE2-P9_ori; Prom_BBa_J23105; STAR_1; Term_T500; MCS
PP_colE2-P9_Tol2_MCS	colE2-P9_ori; Prom_BBa_J23105; STAR_1; Term_T500; Tol2_5_prime; MCS; Tol2_3_prime
PP_colE2-P9_ePB_MCS	colE2-P9_ori; Prom_BBa_J23105; STAR_1; Term_T500; ePB_5_prime; MCS; ePB_3_prime
PP_colE2-P9_SB_MCS	colE2-P9_ori; Prom_BBa_J23105; STAR_1; Term_T500; SB_5_prime; MCS; SB_3_prime
eGFP_cassette	Prom_BBa_J23104; RBS_ BBa_K143021; eGFP_seq_ec; Term_2
pcolE2-P9_eGFP	colE2-P9_ori; eGFP_cassette
PP_colE2-P9_eGFP_noPT	colE2-P9_ori; STAR_1; eGFP_cassette
PP_colE2-P9_eGFP	colE2-P9_ori; Prom_BBa_J23105; STAR_1; Term_T500; eGFP_cassette
PP_colE2-P9_eGFP_Tol2	colE2-P9_ori; Prom_BBa_J23105; STAR_1; Term_T500; Tol2_5_prime; eGFP_cassette; Tol2_3_prime
PP_colE2-P9_eGFP_ePB	colE2-P9_ori; Prom_BBa_J23105; STAR_1; Term_T500; ePB_5_prime; eGFP_cassette; ePB_3_prime
PP_colE2-P9_eGFP_SB	colE2-P9_ori; Prom_BBa_J23105; STAR_1; Term_T500; SB_5_prime; eGFP_cassette; SB_3_prime
STAR_cassette	Prom_BBa_J23105; STAR_1; Term_T500
mCherry_cassette	Prom_CMV; mCherry_seq_hs; STAR_cassette; polyA_BGH
pcolE2-P9_Tol2_MCS	colE2-P9_ori; Tol2_5_prime; MCS; Tol2_3_prime
pcolE2-P9_Tol2_mCherry	colE2-P9_ori; Tol2_5_prime; mCherry_cassette; Tol2_3_prime
parA_induc_lysA	HA_left_lysA; Term_BBa_B1005; Prom_1; RBS_1; parA_seq; Term_1; Prom_BBa_J23108; Tta2_pT181; lysA_seq; Rec_frt_wt; Term_T7; Prom_kanR; KanR_seq; RBS_rhaS_20k; SacB_seq; Rec_frt_wt; Term_bidir; HA_right_lysA
pir_induc_lysA	HA_left_lysA; Term_BBa_B1005; Prom_1; RBS_1; Pir_seq; Term_1; Prom_BBa_J23108; Tta2_pT181; lysA_seq; Rec_frt_wt; Term_T7; Prom_kanR; KanR_seq; RBS_rhaS_20k; SacB_seq; Rec_frt_wt; Term_bidir; HA_right_lysA